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Non-Aβ Component of Alzheimer's Disease Amyloid (NAC) Revisited

NAC and α-Synuclein Are Not Associated with Aβ Amyloid

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α-Synuclein (αSN), also termed the precursor of the non-Aβ component of Alzheimer's disease (AD) amyloid (NACP), is a major component of Lewy bodies and Lewy neurites pathognomonic of Parkinson's disease (PD) and dementia with Lewy bodies (DLB). A fragment of αSN termed the non-Aβ component of AD amyloid (NAC) had previously been identified as a constituent of AD amyloid plaques. To clarify the relationship of NAC and αSN with Aβ plaques, antibodies were raised to three domains of αSN. All antibodies produced punctate labeling of human cortex and strong labeling of Lewy bodies. Using antibodies to αSN(75–91) to label cortical and hippocampal sections of pathologically proven AD cases, we found no evidence for NAC in Aβ amyloid plaques. Double labeling of tissue sections in mixed DLB/AD cases revealed αSN in dystrophic neuritic processes, some of which were in close association with Aβ plaques restricted to the CA1 hippocampal region. In brain homogenates αSN was predominantly recovered in the cytosolic fraction as a 16-kd protein on Western analysis; however, significant amounts of aggregated and αSN fragments were also found in urea extracts of SDS-insoluble material from DLB and PD cases. NAC antibodies identified an endogenous fragment of 6 kd in the cytosolic and urea-soluble brain fractions. This fragment may be produced as a consequence of αSN aggregation or alternatively may accelerate aggregation of the full-length αSN. *(Am J Pathol 1999, 155:1173–1181)*

Since the finding that two mutations in the α-synuclein (αSN) gene are associated with Parkinson's disease (PD),[1,2] several reports have shown that αSN is a major component of Lewy bodies and associated Lewy neurites, pathological hallmarks of Parkinson's disease and dementia with Lewy bodies (DLB).[3–7] αSN has also been found in the neuronal and glial inclusions of multiple system atrophy, Lewy body-like inclusions in the motor neuron disorder amyotrophic lateral sclerosis, and in neuronal inclusions in familial Alzheimer's disease (AD).[8–13] Evidence so far indicates all these cytoplasmic inclusions are filamentous and of similar composition.[14–17] However, αSN is not found in inclusions present in Pick's disease,[17] indicating that αSN is selectively deposited in certain neurodegenerative diseases.

A fragment of αSN was earlier shown to co-purify with AD amyloid. Two peptides isolated after formic acid, CNBr, and protease treatment of AD brain amyloid are termed the non-amyloid component of AD plaques (NAC). They were shown to correspond to residues 61–80 and 81–95 of a larger precursor termed NACP, which was subsequently cloned[18] and found to be a human homolog of the *Torpedo* ray synuclein, which had been previously identified in synaptic vesicle preparations.[19] Synucleins constitute a family of proteins consisting of α, β, and γ SN, now studied in several vertebrates (reviewed[20,21]). α- and β-Synucleins have been shown to be cytoplasmic proteins associated with presynaptic junctions[22,23] and αSN to be the only member associated with intracellular inclusions in neurodegenerative conditions.[3,5,15,24] Antibodies to peptides in the NAC region [rabbit antibody (Ab) X1 to αSN(61–69) and rabbit Ab Y to αSN(81–87)] were reported to label a large proportion of AD plaques.[16,18,25,26] In the present study, we reexamined these findings using antibodies raised to αSN(75–91) of the NAC component.

We have also confirmed the immunoreactivity of neuronal inclusions in dementia with Lewy bodies, and PD

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using rabbit antibodies to N-terminal, NAC, and C-terminal domains of αSN by immunocytochemistry. Western blots of brain homogenates from frozen tissue of these cases were compared with age-matched controls and AD tissues to examine the expression and solubility of αSN and NAC and their relationship with Aβ amyloid.

Materials and Methods

Antibodies

Rabbit polyclonal antibodies were raised to the human αSN N-terminal region (1–18) (Ab 97/5), to the C-terminal domain (116–131) (Ab 97/8), and to the NAC region of αSN (75–91) (Ab 42580). Rabbit antibody Ab 98/13 was raised to β-synuclein (99–113). For preabsorption experiments 10 μg of immunizing peptide was preincubated with 1 to 2 μl of antisera for 16 hours at 4°C in 0.1 ml of PBS, pH 7.4, for immunohistochemistry or in 0.5 ml of TBS-T (50 mmol/L Tris-HCl pH 8.0, 150 mmol/L NaCl with 0.2% Tween 20) for Western blot, before appropriate dilution for use. Mouse monoclonal antibodies (MAb) 1E8 and WO2 recognize Aβ(17–24)[27] and Aβ(5–8),[28] respectively. Rabbit antiserum to human tau was from Dako (Glostrup, Denmark). Synaptophysin mouse MAb SY38 was from Boehringer Mannheim (Mannheim, Germany).

Tissue Collection

Brain tissue was collected at autopsy. Tissue from clinically and pathologically confirmed cases of five DLB, four PD, seven sporadic AD, five DLB/AD, and seven age-matched controls were used in the study. The pathological diagnosis was made according to standard criteria. AD was diagnosed using CERAD criteria.[29] DLB cases were diagnosed using the consensus guidelines[30] when cortical Lewy bodies were seen using ubiquitin immunohistochemistry on the initial screen. A pathological diagnosis of PD was made in conjunction with clinical PD and pathology predominating in the midbrain. For histochemistry, tissues were fixed in 10% formalin in PBS. For Western blot analysis tissues were frozen and stored at −70°C.

Immunocytochemistry

Formalin-fixed tissue from the substantia nigra, hippocampus, and cortex was embedded in paraffin. Sections were treated with 80% formic acid for 5 minutes, treated with 3% hydrogen peroxide for 5 minutes, and incubated in blocking buffer (50 mmol/L Tris-HCl, 175 mmol/L NaCl, pH 7.4, with 20% serum corresponding to species for secondary Ab) before incubation with primary antibody. Ab 97/8 was used at 1:2000, Ab 97/5 at 1:500, Ab 42580 at 1:100, Ab 98/13 1:200, MAb 1E8 was undiluted hybridoma culture supernatant, and rabbit anti-human tau was used at 1:400. Secondary reagents linked to horseradish peroxidase were used and visualized with diaminobenzidine. For double labeling, sections were reacted with an additional secondary antibody conjugated to alkaline phosphatase and developed with 5-bromo-4-chloro-3-indoxyl phosphate and nitro blue tetrazolium chloride (blue) from Dako (K598) or new fuchsin (Dako, K0596) (red). Sections were counterstained with hematoxylin.

Immunofluorescence Labeling

Hippocampal and cortical primary neurons were cultured from embryonic day 15 rats on poly(L)-lysine-coated glass coverslips for 15 days, as previously described.[31] Cells were washed twice with PBS containing 1 mmol/L $CaCl_2$ and 1 mmol/L $MgCl_2$ and fixed with 4% formaldehyde in PBS, pH 7.4, for 15 minutes at room temperature (RT) or for 30 seconds in acetone at −20°C. Cells were then washed twice in PBS. Formaldehyde-fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at RT. Cells were treated with 20% sheep serum in PBS for 10 minutes at RT before incubation with primary Ab diluted in 1% bovine serum albumin in PBS. After washing, cells were reacted with FITC-conjugated sheep anti-rabbit Ig (Amrad, Boronia, Victoria, Australia) or Texas Red conjugated sheep anti-mouse Ig (Amersham, Little Chalfont, England). Double labeling steps were performed sequentially. Coverslips were mounted in 2.6% DABCO (Sigma) in 90% glycerol/10% PBS and imaged with a BioRad 1024 confocal system.

Western Blotting

Brain homogenates were prepared from tissue stored at −70°C by sonication in 1:10 (g/vol) in TBS buffer containing 50 mmol/L Tris-HCl, pH 7.4, 175 mmol/L NaCl, 5 mmol/L EDTA, and the protease inhibitors PMSF (2 mmol/L), aprotinin (2 μg/ml), leupeptin (2 μg/ml), antipain (2 μg/ml), and pepstatin (2 μg/ml). After 5 minutes of centrifugation at 1000 × *g*, the supernatants were centrifuged at 150,000 × *g* for 1 hour at 4°C. This high speed supernatant fraction is termed TBS-soluble. The pellets were rinsed twice in TBS before solubilizing in 5% SDS in TBS and further centrifugation at 150,000 × *g* for 30 minutes. This supernatant was termed the SDS-soluble fraction. The SDS-insoluble pellet was solubilized in 8 mol/L urea/5% SDS in TBS and termed the urea-soluble fraction. Protein concentration was determined in the TBS and SDS soluble fractions using a BCA assay (Pierce, Rockford, IL). Samples were mixed with 2X Laemmli sample buffer containing 10% β-mercaptoethanol and boiled for 5 minutes. 5 μg of protein of TBS fractions, 10 μg of protein of SDS fractions, and 30 μl of urea-soluble material from approximately 30 μg equivalent of frozen tissue were electrophoresed on 10% SDS Tris-tricine polyacrylamide gels and analyzed by Western blot developing with chemiluminescence (ECL, Amersham).[32,33] Western blot analysis of the 1000 × *g* pellet revealed Aβ and αSN but not the 6-kd NAC fragment in all of the samples. The expression levels of αSN in this pellet follows the pattern of the urea fraction, ie, high in DLB/PD cases and minimal amounts in control/AD cases (data not shown). We chose to use 8 mol/L urea/5% SDS to solubilize αSN/NAC ag-

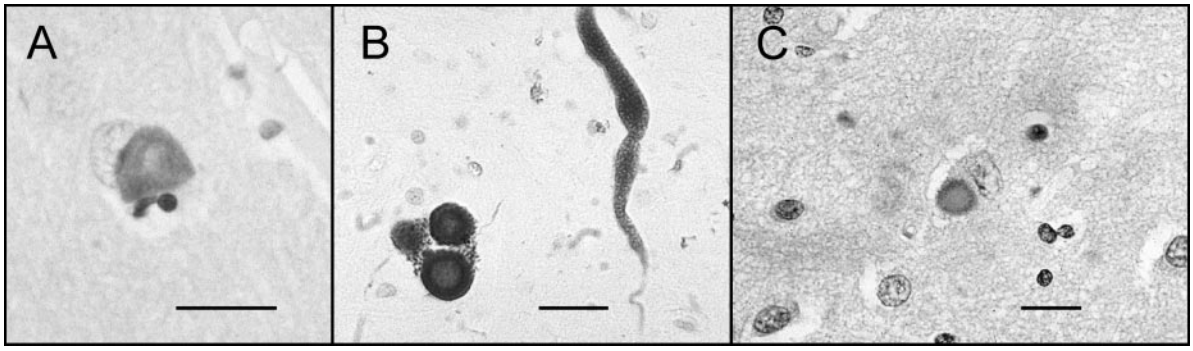

Figure 1. αSN immunoreactivity of neuronal inclusions in DLB brain tissue. A: Labeled Lewy body in cingulate gyrus cortex using N-terminal Ab 97/5. B: Labeled Lewy bodies and neurite in substantia nigra using NAC Ab 42580. C: Labeled Lewy body in cingulate gyrus cortex using C-terminal domain Ab 97/8. Scale bars, 20 μm.

gregates, because in our laboratory we have found that 8 mol/L urea solubilizes amyloid plaques from human AD brain homogenate more efficiently and less variably than formic acid.[34] Under our conditions, materials have been completely solubilized with 8 mol/L urea/5% SDS, as no pellet was left after this step. Furthermore, SDS-insoluble Aβ was also detected with this solubilization method.

Results

Detection of Full-Length αSN in Brain by Immunocytochemistry and Western Blot

To investigate αSN expression, rabbit antibodies were raised to three αSN regions: N-terminal (1–18) Ab (97/5), anti-NAC (75–91) Ab 42580, and the C-terminal domain (116–131) Ab 97/8. This last peptide is most specific for αSN, a region of least homology with other members of the synuclein family.[21] Immunocytochemistry showed that all three antibodies to αSN reacted with a fine punctate pattern for human brain regions rich in synapses and strongly labeled Lewy bodies and Lewy neurites of DLB ($n = 5$) and PD ($n = 4$). Representative labeling of Lewy bodies and a Lewy neurite is shown for cingulate gyrus cortex (Figure 1,A and C) and substantia nigra (Figure 1B) from a DLB case. Antisera to β-synuclein (Ab 98/13) also produced a punctate synaptic type pattern by histochemistry but did not label Lewy body or neurite inclusions (not shown). Preabsorption with immunizing peptides abolished reactivity and the preimmune sera were negative (not shown).

In Western blotting of extracts of human brain homogenates, antibodies to the three regions of αSN reacted with a major band with apparent mobility of 16 kd (Figure 2,A–C), a result consistent with earlier studies.[22,23,35] The β-synuclein Ab 98/13 reacted with a band migrating close to that for αSN as detected by Ab 97/8 (Figure 2D). Preabsorption of antibodies with the immunizing peptides removed reactivity as detected by Western analysis (Figure 2). Preimmune sera were also negative on Western blots.

To investigate further the nature of synaptic reactivity of the antibodies, mature primary rat neurons (cortical and hippocampal) were analyzed for αSN reactivity by immunofluorescence in comparison with the integral synaptic membrane protein synaptophysin. After fixation with formaldehyde, double labeling for the two proteins showed reactivity for all of the αSN Abs and considerable co-localization with synaptophysin as shown for mature hippocampal neurons (Figure 3, A–F) similar to the results reported by Withers et al[36] using a C-terminal αSN MAb. However, not all synaptophysin punctate reactivity was also positive for αSN as indicated in Figure 3 (E and F). It is also of interest that most αSN reactivity was lost after acetone-alone fixation, indicative of a loose association with cellular structures, as shown for αSN labeling with Ab 97/8 (Figure 3H), in contrast to the strong labeling of the integral membrane protein synaptophysin which was retained after acetone fixation (Figure 3G). Cell body labeling was also found for the anti-NAC antibody which was used at a lower dilution and is considered nonspecific, since it was not observed for the other two α-SN antibodies and could not be removed by preabsorption (data not shown). Withers et al[36] also observed nonspecific cell body labeling of cultured hippocampal neurons with αSN antibodies using similar labeling conditions.

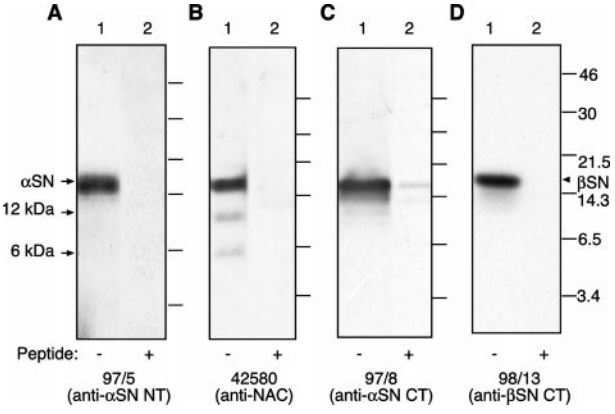

Figure 2. Western blot analysis of synuclein antibody reactivity on human brain homogenate from the cingulate gyrus cortex from DLB case. 10 μg of protein per lane was analyzed on 10% Tris-tricine gel. Antibodies were preabsorbed with peptide as indicated. A: Ab 97/5 at 1:5000 dilution. B: Ab 42580 at 1:1000 dilution. C: Ab 97/8 at 1:20,000. D: Ab 98/13 at 1:1000.

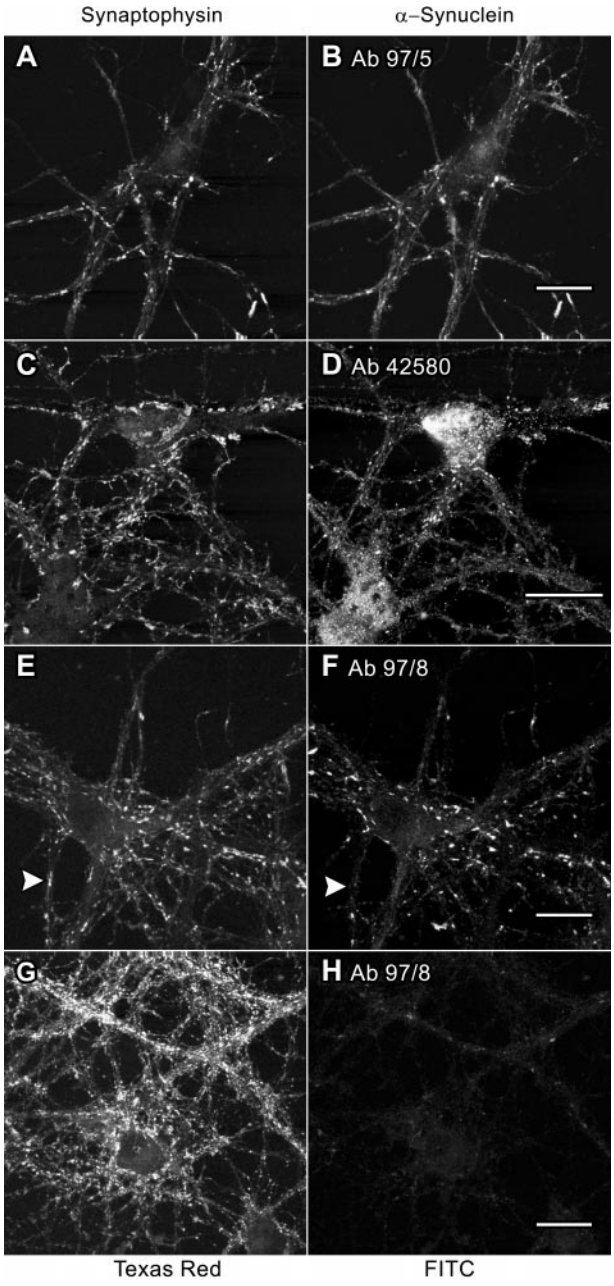

Figure 3. Confocal laser micrographs of double labeling of rat neurons stained for synaptophysin (Texas Red) and αSN (FITC). A, B: Labeling for synaptophysin showed almost complete co-localization with Ab 97/5 diluted 1:1000 after formaldehyde fixation. C, D: Labeling with NAC Ab 42580 at 1:100 after formaldehyde fixation also showed a punctate labeling with a higher apparent background over neuronal cell bodies, and there was considerable overlap with synaptophysin labeling. E, F: Labeling for synaptophysin showed considerable co-localization with Ab 97/8 diluted 1:1000 after formaldehyde fixation; some synaptophysin-positive punctate staining (arrowheads) did not have corresponding synuclein staining. G, H: Labeling after acetone fixation alone showed strong synaptophysin labeling but only very weak synuclein labeling (H). Scale bars, 20 μm.

NAC Is Not Found in Association with Most Aβ Amyloid Plaques of AD Brain

Earlier studies have indicated that antibodies to the NAC fragment of αSN label AD amyloid plaques.[16,18,25,26] Since these reports have not been confirmed with independently prepared antibodies, we reexamined this question using antibodies raised to the central NAC domain using Ab 42580 on AD cortical and hippocampal sections. We found no evidence of NAC labeling in plaques of cortical or hippocampal tissue sections from AD only cases (Figure 4, B and D). Serial sections labeled in parallel for Aβ revealed numerous plaques (Figure 4, A and C). Our other αSN antibodies which strongly labeled synapses and Lewy body inclusions (Ab 97/5 and Ab 97/8) also showed no plaque labeling in AD only cases (not shown).

Although previous reports of anti-NAC labeling of Aβ plaques used vibratome slices, we do not consider our results reflect differences in processing, since Takeda et al[16] reported similar results for anti-NAC labeling of paraffin sections and vibratome slices.

αSN Deposits Are Occasionally Associated with the Periphery of Aβ Plaques and Tau-Positive Neurofibrillary Tangles

In cases with mixed AD and DLB pathology the distribution of Lewy bodies was similar to that of DLB cases. In the cortex αSN immunoreactivity in and around Aβ plaques was not seen; however, in sections from the hippocampus of DLB/AD double staining for αSN (Ab 97/8) and Aβ (MAb 1E8) showed strong αSN labeling in the CA1 region in dystrophic globular neurites (Figure 5A) at the periphery of Aβ-positive plaques. There were also clusters of αSN-positive globular neuritic structures not in direct association with Aβ plaques (Figure 5B). These neuritic structures were also labeled with the NAC antibody (not shown) which indicated that full-length αSN and possibly also the NAC fragment may be aggregating as neuritic inclusions that may be associated only occasionally with Aβ plaques. These neuritic αSN-positive clusters are similar to those described in AD hippocampus by Munoz and Wang.[37]

Double staining for αSN and tau in the same cases revealed occasional association of αSN inclusions within cells that were also strongly reactive for intraneuronal tau (Figure 5C) as well as close association of αSN-positive dystrophic neuritic structures with tau-positive deposits (Figure 5D). Close expression of tau immunoreactive neurofibrillary tangles and αSN reactivity was also shown in DLB hippocampus by Iseki et al.[38]

Expression and Solubility of αSN and NAC in Human Brain Homogenate Fractions

To investigate the solubility of αSN and its fragments differential extraction procedures were carried out on brain homogenates. TBS homogenates were centrifuged at 150,000 × *g* to generate a cytosolic TBS-soluble fraction and particulate membrane fraction (TBS-pellet). The pellets were then solubilized with 5% SDS and centrifuged at 150,000 × *g* to generate an SDS-soluble fraction. The SDS-insoluble pellet was subsequently solubilized with urea to generate the urea-soluble fraction.

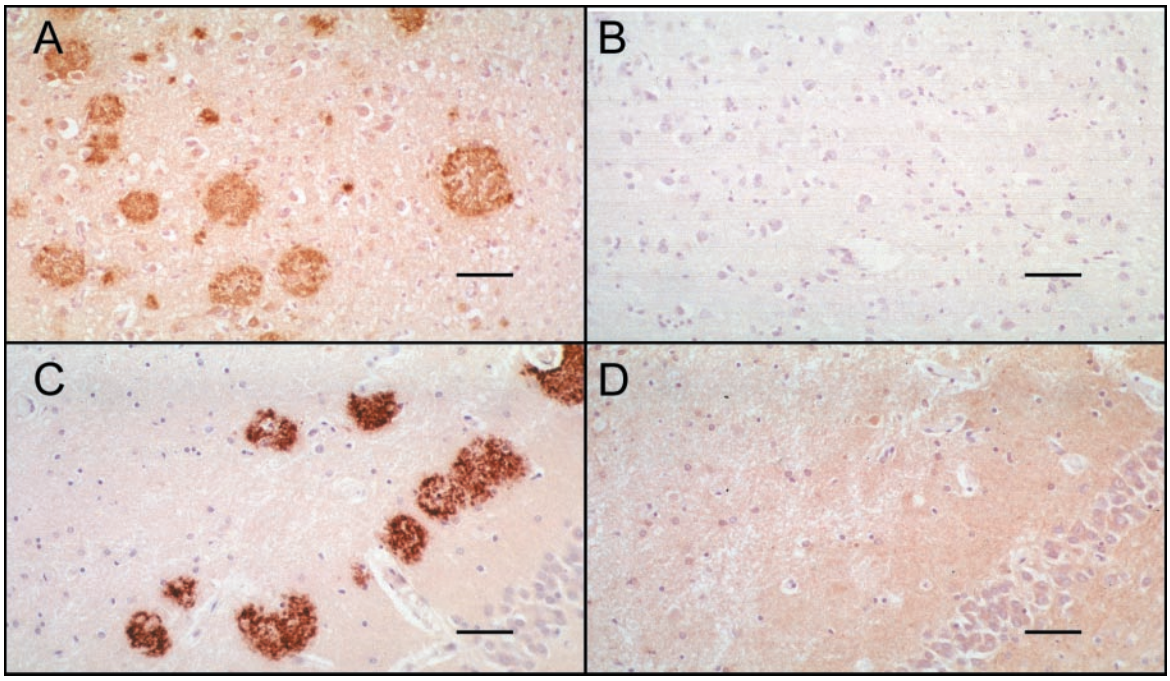

Figure 4. Comparison Aβ and NAC antibody immunoreactivities in amyloid plaques in AD brain sections. A: AD frontal cortex stained with Aβ MAb 1E8 shows numerous plaques. B: A serial section from the same region as used in A stained in parallel with NAC Ab 42580 showed no NAC plaque labeling. C: AD hippocampus CA2 region stained with Aβ MAb 1E8 revealed numerous Aβ-positive plaques. D: The same region as used in C showed no plaque labeling with NAC Ab 42580. Scale bars, 50 μm.

Western blot analysis of the αSN expression in TBS-soluble extracts of human brain cingulate gyrus cortex by Western blotting showed similar reactivity from cases with DLB, PD, AD, and controls (Figure 6A) using C-terminal domain Ab 97/8. AD cases (Figure 6A, lanes 7 and 8) had apparently less αSN which maybe indicative of neuronal synaptic loss. Antibody to the central hydrophobic domain of αSN(75–91), NAC Ab 42580, also de-

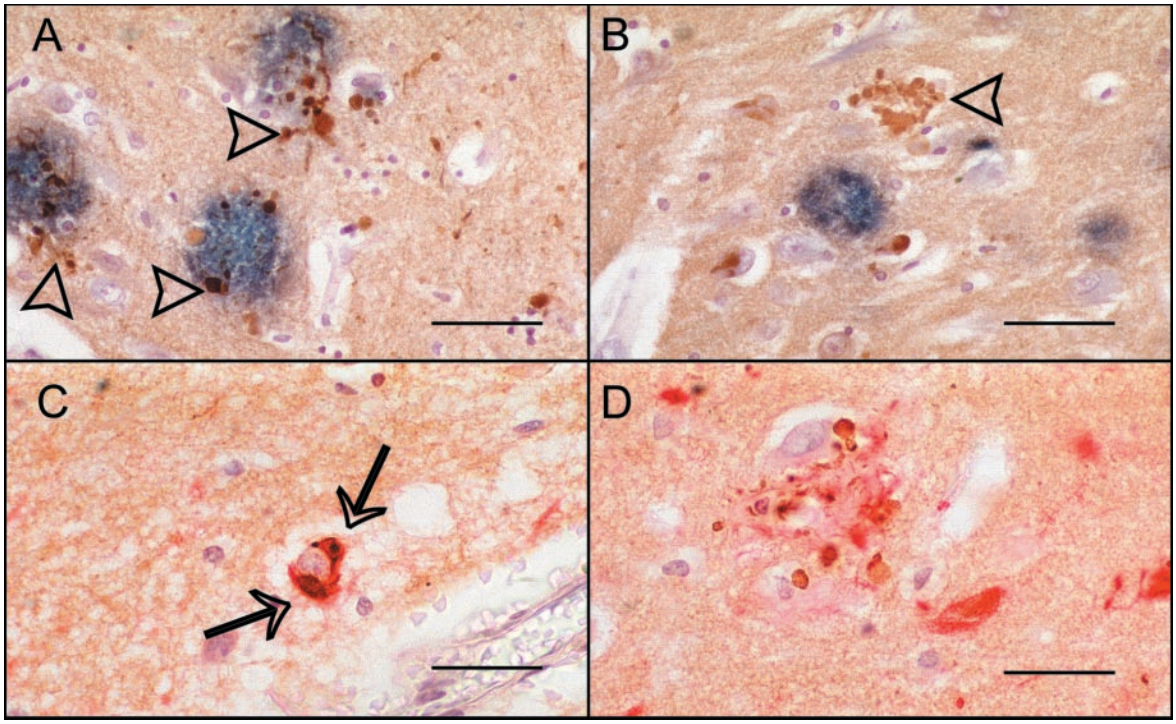

Figure 5. Double labeling of CA1 region of hippocampus sections from DLB/AD case. A, B: αSN labeling (Ab 97/8) in brown and Aβ labeling (MAb 1E8) in blue shows αSN-positive globular neuritic structures surrounding Aβ-positive plaques (arrowheads in A), and αSN-positive globular clusters separate from a nearby Aβ-positive plaque. C, D: αSN labeling (Ab 97/8) in brown and tau labeling in red shows αSN-positive inclusions in neuron with tau-positive intraneuronal staining (arrows in C) and close association of globular αSN-positive neuritic processes with tau-positive structures (in D). Scale bars, 50 μm.

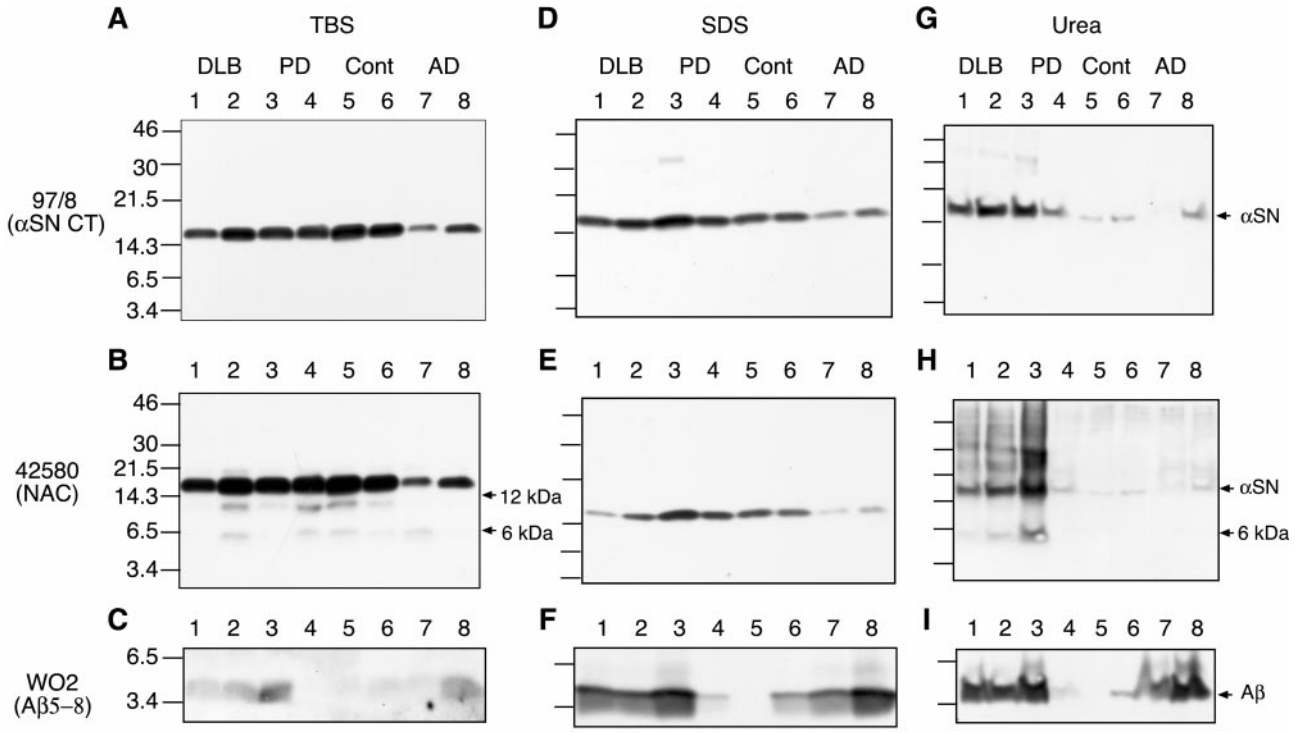

Figure 6. Western blot analysis of αSN expression in human brain fractions extracted from cingulate gyrus cortex of 2 cases each of DLB, PD, control, and AD. 5 μg of protein TBS-soluble, 10 μg of protein SDS-soluble, and 30 μl of urea-soluble fractions were applied per lane and run on separate gels. Due to slight gel variation separate markers were used in each gel as a reference. Blots were incubated with Ab 97/8 at 1:20,000 and exposed to ECL film for 1 minute, Ab 42580 at 1:2000 and exposed to ECL film for 5 minutes, or MAb WO2 at 1 μg/ml and exposed to film 1 minute (SDS) and 5 minutes (TBS).

tected full-length αSN and a less abundant putative NAC fragment of about 6 kd. The presence of the soluble 6-kd NAC was not apparently related to the disease process. Another fragment of about 12 kd was also detected (Figure 6B).

Incubation of Ab 97/8 and Ab 42580 with the SDS-soluble fractions revealed detection of full-length αSN (Figure 6, D and E) but no detection of the 6-kd NAC fragment (Figure 6E). Analysis of the SDS-insoluble pellet which was solubilized by urea showed marked differences between brain samples. DLB and PD cases showed the most αSN reactivity (Figure 6G) with low amounts in control brains and with variable amounts from low to an intermediate amount in AD cortex samples. Immunoreaction with the NAC antibody again detected full-length αSN (Figure 6H) as well as detection of the NAC fragment in the DLB cases and some PD cases (Figure 6H). Significant amounts of apparently aggregated αSN and/or NAC were detected in urea-soluble fractions, especially with the NAC antibody as evidenced by bands of slower mobility (ie, higher molecular weights) in the DLB and some PD cases (Figure 6H). The 6-kd band detected by the NAC antibody in both the TBS (soluble fraction) and the insoluble urea fractions (Figure 6, B and H) are similar as they both run just below the 6.5-kd molecular weight marker and ran with the same electrophoretic mobility when these fractions were run on the same gel (data not shown). Ab 98/13 staining of the urea-soluble fractions revealed no reactivity indicating that β-synuclein is not aggregating like the αSN (not shown).

Examination of Aβ in the brain fractions revealed weak reactivity in the TBS-soluble fraction (Figure 6C). There were significant amounts of Aβ associated with the particulate pellet which was solubilized by SDS (SDS-soluble Aβ) (Figure 6F), and considerable SDS-insoluble but urea-soluble Aβ (Figure 6I) in DLB, one PD, both AD, and negligible amounts in one PD and the controls.

Discussion

Rabbit antibodies generated to the N-terminal domain, central hydrophobic NAC region, and C-terminal domain of αSN all reacted with Lewy body inclusions in DLB brain sections, reacted by Western blotting in brain homogenates with a major product of 16 kd, and also by immunofluorescence at synaptic sites indicating that all epitopes for these antibodies and most likely full-length αSN are expressed at these sites.

These results confirm previous studies which primarily used N- and C-terminal region antibodies, indicating that αSN is a major component of Lewy bodies.[3–7] The relative mobility on the 10% Tris-tricine gel system for αSN of apparent 16 kd is closer to the predicted molecular weight of the 140 amino acid protein than 19 kd reported previously for samples resolved on Tris-glycine gel systems.[18,22,24] We have used the Tris-tricine system for better resolution of smaller peptides such as Aβ[32,33] and used it in this study for improved detection of the NAC peptide. These gels did not resolve a clear difference

between the mobility of α- and β-synuclein (134 amino acids) as shown previously.[24]

Although Uéda et al first described isolation of NAC peptide associated with AD brain amyloid in 1993[18] there has been little further information to confirm or characterize this fragment in human brain. Western blot analysis in Figure 2B and Figure 6, B and H, shows that the NAC Ab 42580 detected the full-length αSN as well as a 6-kd fragment. This size for the putative NAC is larger than anticipated for a fragment of 35 amino acids. Since NAC was originally isolated using CNBr and protease digestion, the size predicted for the putative NAC may underestimate the size of an endogenous fragment found in the brain. The 6-kd fragment detected in our system appears to be longer than the suggested αSN(61–95) residues.

By immunocytochemistry the NAC antibody failed to show labeling of Aβ plaques in brain sections from patients with pathologically confirmed AD or of the cores of plaques from mixed DLB/AD cases. In mixed DLB/AD cases αSN immunoreactivity was found at the periphery of some Aβ plaques and as clusters of bulbous neuritic processes in only a limited region of the hippocampus, the CA1 region. We also noted dense αSN immunoreactive Lewy neurites localized to the CA2 hippocampal region of all DLB and mixed DLB/AD cases as has been reported previously.[15,17] Lewy neurites in this region have been noted as a distinguishing feature of DLB and PD.[39,40] Thus all αSN immunoreactivity appeared to be intracellular in contrast to the extracellular deposition of Aβ amyloid plaques, which is suggestive of independent insoluble accumulation of these proteins.

Western blot analysis also showed lack of correlation between expression of Aβ and NAC or between Aβ and αSN detection in TBS-soluble fractions or urea-soluble fractions. Especially in the AD cases studied, urea-soluble NAC or αSN did not accumulate with urea-soluble Aβ. However, in the Lewy body disease cases there was significant expression of αSN, NAC, and Aβ in the urea-soluble fractions. Cases 1 to 3 did not have sufficient neuritic Aβ plaque morphology to be classified as AD pathology; however, they had moderate numbers of diffuse plaques and cases 2 and 3 also had a congophilic angiopathy. One PD case examined had very few diffuse plaques and low co-expression of αSN, NAC, and Aβ in the urea-soluble fraction (case 4).

It is only relatively recently that DLB has been distinguished as a major cause of dementia. When DLB and AD co-exist, the incidence of Lewy bodies is difficult to distinguish from neurofibrillary tangles in cortical regions since both label with ubiquitin antibody which has been used to date to detect Lewy bodies.[40,41] An improved assessment of the incidence and relationship of DLB and mixed DLB/AD will now be possible with the advent of αSN antibodies to identify more accurately Lewy bodies in routine pathological assessment.

We found immunocytochemical labeling patterns with the NAC antibody indistinguishable from the other αSN antibodies as also reported for another recently generated NAC antibody tested on ischemic gerbil hippocampus.[42] Examination of double labeling for αSN and synaptophysin reactivity on cultured hippocampal rat neurons confirmed a synaptic localization for αSN that was easily disrupted by acetone fixation, suggesting a loose association with cellular structures. This was consistent with αSN solubility properties and earlier localization studies.[35,36] Recent *in vitro* studies indicate synthetic αSN associates with small lipid vesicles by electrostatic interaction.[43] Hsu et al[44] showed that αSN is expressed later in murine development than synaptophysin and progressively moves from being solely cytosolic to more particulate throughout development, consistent with our Western blot data that show that a pool of αSN is found in the membrane particulate fraction (Figure 6, D and E).

Comparison of immunoreactivity by Western blotting of extracts of human brain homogenates indicated that most αSN is expressed in the TBS-soluble cytosolic fraction generated from the 150,000 × *g* centrifugation step in different disease states. Some reduction may occur in AD with loss of neuronal synapses. The 6-kd putative NAC is also present in low amounts in all of the TBS-soluble samples. The levels of both soluble αSN and soluble 6-kd fragment show no correlation with disease state. The presence of significant amounts of αSN in the SDS fraction is consistent with our immunofluorescence data and other reports that αSN can associate with small lipid vesicular structures, since the SDS fraction is derived from the TBS-insoluble fraction and comprises membranous structures. Since there is no detectable 6-kd NAC fragment in the SDS fraction, this indicates that the fragment is probably a normal soluble breakdown product of αSN and not associated with membrane/vesicle structures. Marked differences were seen in the urea-soluble brain fractions, with most αSN and 6-kd immunoreactivity in the Lewy body disorder cases of DLB and some of the PD cases, indicating that less soluble and aggregated αSN and the putative NAC accumulates in these disorders. The NAC fragment of 6 kd and higher molecular weight aggregates particularly detected with the NAC Ab 42580 indicate the SDS-insoluble material forms aggregates which could form the Lewy bodies and Lewy neurites and is disease-related. These aggregates are not recognized by the β-synuclein antibody consistent with the negative immunoreactivity of β-synuclein antibody for Lewy bodies in brain sections by immunocytochemistry. Fractions containing αSN of reduced solubility have also been reported for neurodegenerative cases of multiple system atrophy[24] and familial AD cases that contained many Lewy body inclusions.[10] Truncated αSN polypeptides of 14 to 16 kd as well as full-length αSN and aggregates were detected using a monoclonal antibody by Baba et al in formic acid extracts of purified Lewy bodies.[3]

The results presented here indicate the urea-soluble fraction may represent aggregated αSN deposits from the Lewy bodies and neurites of these cases and may include aggregated NAC. NAC may therefore be a comparatively protease-resistant core of the protein which is particularly prone to aggregation, as shown previously with NAC synthetic peptide.[25,45–47] NAC may accumulate as a natural breakdown product of αSN aggregation or NAC aggregates may contribute to further aggregation of the full-length αSN, rather than seeding aggregation of

the Aβ amyloid peptide as had been proposed earlier. The mechanism underlying αSN aggregation will be important for understanding the role of αSN in neurodegeneration.

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